

Novel Opioid Peptides for the Treatment of Pain

Field of the Invention

The present invention is directed to opioid-like peptide compounds that exhibit peripheral analgesic activity and selectivity for the μ subtype of opioid receptors.

5 Background of the Invention

The development of new and effective agents for alleviating the peripheral pain of patients is a major objective of medicine. In this regard, it has recently been reported that opiates have significant peripheral analgesic activity (Barber *et al.*, *Med. Res. Rev.* 12:525 (1992); and Stein, *Anesth. Analg.* 76:182 (1993)). Quaternary salts of known centrally acting opioid alkaloids
10 have been used as pharmacological probes to distinguish between peripheral and central analgesic responses. These quaternary salts have a permanent positive charge and show restricted penetration of the blood-brain barrier (see Smith, *et al.*, *Life Sci.* 31:1205 (1982); Smith, *et al.*, *Int. J. Tiss. Reac.* 7:61 (1985); Lorenzetti, *et al.*, *Braz. J. Med. Biol. Res.* 15:285 (1982); Brown, *et al.*, *Neuropharmacol.*, 24:181 (1985); Bianchi *et al.*, *Life Sci.* 30:1875 (1982); and Russel, *et al.*, *Eur. J. Pharmacol.*, 78:255 (1982)).
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Many endogenous peptides of mammalian and amphibian origin (*e.g.* the endorphins) bind to opioid receptors and elicit an analgesic response similar to classic narcotic opiates. This led to the hope that these peptides might be produced commercially and administered to patients to relieve pain. Unfortunately, administration of opioid peptides to mammals has been shown to
20 affect the central nervous system. For example, it has been reported that TAPP (H-Tyr-D-Ala-Phe-Phe-NH₂) exhibits antinociceptive properties both peripherally and centrally (Schiller, *et al.*, Proceedings of the 20th European Peptide Symposium (1988)). Because side effects such as respiratory depression, tolerance, physical dependence capacity, and precipitated withdrawal syndrome are caused by nonspecific interactions between opiates and central nervous system
structurally modify opioid peptides to lessen CNS penetration. Highly polar analogues of enkephalins and dermorphins have been prepared which retain high antinociceptive activity but

show limited central nervous system penetration (see, Follenfant, *et al.*, *Br. J. Pharmacol.* 93:85 (1988); Hardy, *et al.*, *J. Med. Chem.*, 32:1108 (1989)).

In order to be effective, an analgesic agent must act at the particular opioid receptors responsible for providing pain relief. Three different types of opioid receptors have been identified. The first type, δ , exhibits a preference for enkephalin-like peptides. The second, μ , shows enhanced selectivity for morphine and other polycyclic alkaloids. Finally, κ -type receptors exhibit equal affinity for either group of ligands and preferential affinity for dynorphin. In general, it appears that the μ receptors are the ones primarily responsible for mediating analgesic effects, although the δ - and the κ -receptors may also play a role.

Each opioid receptor, when bound by opiate, causes a unique biological response. When an opiate activates more than one receptor type, the biological response associated with each is induced, thereby producing side effects. Thus, an ideal peptide for alleviating pain would both act specifically on μ -type opioid receptors and not cross the blood-brain barrier. The present invention is directed to peptides that approach this ideal.

Summary of the Invention

The present invention is directed to peptides that exhibit both specificity and selectivity for binding to μ -opioid receptors. As used herein, a peptide has specificity for the μ -type receptor if it has a greater affinity (*i.e.* lower binding inhibition constant, K_i) for this receptor than for either δ - or κ -type receptors. The term "selectivity" refers to the ability of an opiate or opioid peptide to discriminate among several opioid receptors and to bind to only one. The selectivity of an opioid peptide is indicated by the ratio of binding inhibition constants. For instance, the ratio of binding inhibition constants, $K_{i\delta}/K_{i\mu}$, is a value that may be used to measure selectivity. This ratio represents the relationship of the affinities for binding to the μ and δ receptors. A higher value for this ratio indicates a greater preference of ligand to bind with the μ receptor over the δ receptor. One conventional opioid peptide analog, H-Tyr-D-Ala-Gly-Phe(NMe)-Gly-ol

This fractional value reflects a pronounced affinity for the δ receptor over the μ receptor.

Figure 2 shows dose response curves for BCH2463 in the phenylquinone-induced writhing assay (in the mouse sc.) $ED_{50} = 0.5 \text{ mg/kg}$ at 20 minutes post administration.

Figure 3 lists comparative analgesic time course of BCH1774 and BCH2463 in the phenylquinone-induced writhing assay (mouse s.c.)

5 Figures 4A, 4B, 5A, 5B, 7A, and 7B illustrate the inhibitory effect of compounds of formula (B) in two different hot-plate assays.

Figure 6 illustrates the inhibitory effect of H-Tyr-D-Ala-Phe-Phe-NH₂ in a hot-plate assay.

10 Figure 8A and 8B illustrate the inhibitory effect of compounds of formula (B) in the tail-flick assay.

Definitions and Abbreviations

The following common abbreviations are used throughout the specification and in the claims:

Abu - aminobutyric acid	Aib - aminoisobutyric acid
15 Ala - alanine	Arg - arginine
BOP-benzotriazolyl-N-oxy-tris(dimethylamino) phosphordumhexafluorophosphate	Chl - cyclohomoleucine
Cle - cycloleucine	Cys (Bzl) - cysteine (benzyl)
DMF-dimethylformamide	Dmt - 2'6'-dimethyltyrosyl
20 FMOC -9-fluorenylmethyloxycarbonyl	Gln - glutamine
Glu - glutamic acid	Gly - glycine
GPI - guinea pig ileum	His - histidine
Met - methionine	MVD - mouse vas deterens

	Nal-1', or 2'-naphthylalanine	Nle - norleucine
	Nva - norvaline	PBQ -phenyl- ρ -benzoquinone
	pF - parafluoro	Phe - phenylalanine
	Phe(pF) - parafluoro phenylalanine	Phg - phenylglycine
5	Pmc-2,2,5,7,8 pentamethylchroman-6-sulfonyl	Pro - proline
	Ser - serine	TAPP - H-Tyr-D-Ala-Phe-Phe-NH ₂
	tBU-tert-butyl	TFA-trifluoroacetic acid
	Thr - threonine	Tic - tetrahydroisoquinoline-3-carboxylic acid
	Trp - tryptophan	TSPP - H-Tyr-D-Ser-Phe-Phe-NH ₂
10	Tyr - tyrosine	∇ - cyclopropyl

The terms "amino acid," and "aromatic amino acid," as used herein, include naturally occurring amino acids as well as non-natural amino acids, their derivatives, and analogues, commonly utilized by those skilled in the art of chemical synthesis and peptide chemistry. Also analogues of TAPP in which the phenylalanine is para-substituted at position 4 with a nitro or azido residue are included. A list of non-natural and non-proteogenic amino acids may be found in "The Peptide", vol 5, 1983, Academic Press, Chapter 6 by D.C. Roberts and F. Vellaccio which is incorporated herein by reference. Examples of aromatic amino acids include tyrosine, tryptophan, phenylglycine, histidine, naphthylalanine, tetrahydroisoquinoline-3carboxylic acid and benzylcysteine. Other examples of aromatic amino acids include phenylalanine substituted on its aromatic ring with, for example, CH₃, C₂H₅, F, Cl, Br, NO₂, OH, SH, CF₃, CN, COOH, and CH₂COOH. These examples are intended to be exemplary only and are not intended to limit the invention in any way.

The term "ED₅₀" as shown in Table 1 for the PBQ writhing assays is defined as the dose of drug which induces a 50% reduction in the number of writhes observed compared to the

product analysis.

The term "interposed amide bond mimetic" is a bond in which the carbonyl group and the NH group of an amide bond are interchanged.

The term " K_i " is the binding inhibition constants. The term " $K_{i\delta}/K_{i\mu}$ " is a value used to indicate selectivity. This ratio represents the relationship of the affinities of opioid peptides for binding to the μ and δ -receptors.

The term "R-configuration" refers to the three dimensional arrangement of substituents around a chiral element. A general system for designating absolute configuration is based upon a priority system which is well-known to persons skilled in the art and is briefly described hereafter. Each group attached to the chiral center is assigned a number according to priority. The molecule is viewed from the side opposite the lowest priority. The configuration is specified "R" if the eye proceeds in a clockwise direction when traveling from the group of highest priority to the group of lowest priority.

The term "residue" when applied to an amino acid, means a radical derived from the corresponding amino acid by removing the hydroxyl of the carboxyl group and one hydrogen from the amino group.

Detailed Description of the Invention

A. Compounds of Formula (A)

In its first aspect, the compounds of the present invention are defined by formula (A) and the associated definition of terms set forth in the Summary section above. For these compounds, as well as for each group of preferred compounds discussed below, there is a preferred subgroup in which X is H.

One group of preferred compounds is represented by formula (A) and derivatives and analogues thereof, wherein R_2 is an amino acid residue having the R-configuration, aminoisobutyryl residue, R_1 is a p-aminophenyl and R_3 and R_4 are p-phenyl and R_5 and R_6 are different and are selected from the group consisting of phenylalanine and tryptophan.

Another group of preferred compounds is represented by formula (A) and derivatives and analogues thereof, wherein Q is an amide bond or an interposed amide bond mimetic of the formula Q_1-Q_2 wherein Q_1 , is selected from the group consisting of CH_2 , $CHOH$, $C=O$, $C=S$, and $CH=$, and Q_2 is selected from the group consisting of CH_2 , NH , S , SO , SO_2 , O and $CH=$ with the proviso that when Q_1 , is $CH=$, then Q_2 is $CH=$.

Further preferred compounds are represented by formula (A) and derivatives and analogues thereof, wherein, Y and Z are H; R_3 and R_4 are independently an aromatic amino acid; and R_2 is an amino acid having the R-configuration, aminoisobutyric acid, cyclopropylalanine, cyclohomoleucine or cycloleucine, with the proviso that when R_1 is a tyrosyl residue, and R_2 is D-alanine, then R_3 and R_4 are different and are selected from the group consisting of phenylalanine and tryptophan. In one subset of these preferred compounds, Q is an amide bond or an interposed amide bond mimetic of the formula Q_1-Q_2 wherein Q_1 , is selected from the group consisting of CH_2 , $CHOH$, $C=O$, $C=S$, and $CH=$, and Q_2 is selected from the group consisting of CH_2 , NH , S , SO , SO_2 , O and $CH=$ with the proviso that when Q_1 , is $CH=$, then Q_2 is $CH=$.

Other preferred compounds are represented by formula (A) and derivatives and analogues thereof, wherein, R_2 is an amino acid having the R-configuration, aminoisobutyric acid, cyclopropylalanine, cyclohomoleucine or cycloleucine with the proviso that R_2 is not D-alanine; and R_3 and R_4 are phenylalanyl residues.

Still, other preferred compounds are represented by formula (A) and derivatives and analogues thereof, wherein, R_1 is a tyrosyl residue; R_2 is selected from the group consisting of D-norvaline, D-serine, and D-arginine; R_3 and R_4 are phenylalanyl residues; and Q is a peptide bond.

Another group of preferred compounds is represented by formula (A) and derivatives and analogues thereof, wherein, Y and Z are independently selected from the group consisting of H, an aromatic amino acid, an aromatic aliphatic amino acid, and an aliphatic amino acid residue having the R-configuration; with the proviso that when R_2 is D-alanine, R_1 is a tyrosyl

residue, and Y and Z are H, then R_3 and R_4 are independently selected from the group consisting of phenylalanine, and tryptophan, but are not the same, Q is an amide bond or an interposed amide bond mimetic of the formula Q_1-Q_2 wherein Q_1 is selected from the group consisting of CH_2 , $CHOH$, $C=O$, $C=S$, and $CH=$, and Q_2 is selected from the group consisting of CH_2 , NH , S , SO , SO_2 , O , and $CH=$ with the proviso that Q_1 is $CH=$, then Q_2 is $CH=$.

Additional preferred compounds are represented by formula (A) and derivatives and analogues thereof, wherein, Y and Z are H; R_1 is a tyrosyl residue, a 2', 6'-dimethyltyrosyl residue, or an analogue or derivative thereof; R_3 and R_4 are independently an aromatic amino acid; R_2 is an amino acid having the R-configuration; with the proviso that when R_2 is D-alanine, and R_1 is a tyrosyl residue, then R_3 and R_4 are independently selected from the group consisting of phenylalanine and tryptophan, but are not the same; Q is an amide bond or an interposed amide bond mimetic of the formula Q_1-Q_2 wherein Q_1 is selected from the group consisting of CH_2 , $CHOH$, $C=O$, $C=S$, and $CH=$, and Q_2 is selected from the group consisting of CH_2 , NH , S , SO , SO_2 , O , and $CH=$, with the proviso that when Q_1 is $CH=$, then Q_2 is $CH=$.

Another group of preferred compounds is represented by formula (A) and derivatives and analogues thereof, wherein, Y and Z are H; R_1 is a tyrosyl residue, 2', 6'-dimethyltyrosyl residue, or an analogue or derivative thereof; R_2 is an amino acid having the R-configuration with the proviso that R_2 is not D-alanine; R_3 and R_4 are phenylalanyl residues; Q is an amide bond or an amide bond mimetic of the formula Q_1-Q_2 wherein Q_1 is selected from the group consisting of CH_2 , $CHOH$, $C=O$, $C=S$, and $CH=$, and Q_2 is selected from the group consisting of CH_2 , NH , S , SO , SO_2 , O , and $CH=$, with the proviso that when Q_1 is $CH=$, then Q_2 is $CH=$.

The most preferred compounds are represented by formula (A) and derivatives and analogues thereof, wherein, Y and Z are H; R_1 is a tyrosyl residue; R_2 is selected from the group consisting of D-norvaline, D-serine, and D-arginine; R_3 and R_4 are phenylalanyl residues, and Q is a peptide bond.

Preferred individual preferred compounds are H-Tyr-Aib-Phe-Phe-NH₂;

- H-Tyr-D-Nle-Phe-Phe-NH₂;
 H-Tyr-D-Ala-Phe-2'-Nal-NH₂;
 H-Tyr-D-Ala-D-Phe-Phe-NH₂;
 H-Tyr-D-Ala-Phe(4NO₂)-Phe(4NO₂)-NH₂;
 5 H-Tyr-D-Ala-Phe-Tic-NH₂;
 H-Tyr-D-Ala-Phe-Phe(NMe)-NH₂;
 H-Tyr-D-Ala-Phe-1'-Nal-NH₂;
 H-Tyr-D-Ala-Trp-Phe-NH₂;
 H-Tyr-D-Ala-Phe-Trp-NH₂;
 10 H-Tyr-∇Ala-Phe-Phe-NH₂;
 ∇CH₂-Tyr-D-Ala-Phe-Phe-NH₂;
 H-Tyr-D-Nle-Phe-Trp-NH₂;
 H-Tyr-D-Nle-Phe-2'-Nal-NH₂;
 H-Tyr-D-Nle-Trp-Phe-NH₂;
 15 H-Tyr-D-Ala-Trp-2'-Nal-NH₂;
 H-Tyr-D-Nle-Trp-2'-Nal-NH₂;
 H-Tyr-D-Nle-Trp-Trp-NH₂;
 H-Tyr-D-Nva-Phe-Phe-NH₂;
 H-Tyr-D-Ser-Phe-Phe-NH₂;
 20 H-Tyr-D-Val-Phe-Phe-NH₂;
 H-Tyr-D-Leu-Phe-Phe-NH₂;
 H-Tyr-D-Ile-Phe-Phe-NH₂;
 H-Tyr-D-Abu-Phe-Phe-NH₂;
 H-Tyr-Chl-Phe-Phe-NH₂;
 25 H-Tyr-Cle-Phe-Phe-NH₂;
 H-Tyr-D-Arg-Phe-Phe-NH₂;
 H-Tyr-D-Cys-Phe-Phe-NH₂;
 H-Tyr-D-Thr-Phe-Phe-NH₂;
 H-Tyr-D-Ala-Phe-Phe-Phe-NH₂ trifluoroacetate
 H-Tyr-D-Ala-Phe-Phg-NH₂ trifluoroacetic acid salt;

- H-Tyr-D-Arg-Phe-Hph-NH₂ bis-trifluoroacetic acid;
 H-DMT-D-Ala-Phe-Phe-NH₂ trifluoroacetic acid;
 H-D-DMT-D-Ala-Phe-Phe-NH₂ trifluoroacetic acid salt;
 H-Tyr-D-Ala-Phe-Hph-NH₂ trifluoroacetic acid salt;
 5 H-Tyr-D-Ala-Phe-Cys(Bzl)-NH₂ trifluoroacetic acid salt;
 H-Tyr-D-Arg-Hph-Phe-NH₂ bis-trifluoroacetic acid salt;
 H-Tyr-D-Arg-Phg-Phe-NH₂ bis-trifluoro acetic acid salt;
 H-Tyr-D-Ala-Phe-Phe-CH₂OH hydrochloride salt;
 H-Tyr-D-Ala-Hph-Phe-NH₂ trifluoroacetic acid salt;
 10 H-Tyr-D-Met-Phe-Phe-NH₂ trifluoroacetic acid salt;
 H-Tyr-D-Arg-Phe-D-Phe-NH₂ bis-trifluoroacetic acid salt;
 H-Tyr-D-Ala-Phg-Phe-NH₂ trifluoroacetic acid salt;
 H-Tyr-(D)-Ala-(D)-Phg-Phe-NH₂ trifluoroacetic acid salt;
 H-Tyr-D-Arg-Phe-Phe(pF)-NH₂ bis-trifluoroacetic acid salt;
 15 H-Tyr-D-Arg-Phe-D-Phe(pF)-NH₂ ditrifluoroacetic acid salt;
 H-Tyr-D-Ala-Phe-Phe(pF)-NH₂ trifluoroacetic acid salt; and
 H-Tyr-D-Ala-Phe-D-Phe(pF)-NH₂ trifluoroacetic acid salt.

The most preferred compounds for use in pharmaceutical compositions and in methods
 of treating patients for pain are: H-Tyr-D-Nva-Phe-Phe-NH₂; H-Tyr-D-Ser-Phe-Phe-NH₂; and
 20 H-Tyr-D-Arg-Phe-Phe-NH₂. Of these, the most highly preferred is H-Tyr-D-Arg-Phe-Phe-NH₂.

B. Analgesic Activity of the Compounds of Formula A

A number of tetrapeptides based on formula (A) have been prepared and evaluated as
 opioid receptor ligands and systemically acting analgesic agents. These compounds are listed
 in Table 1 along with their respective binding inhibition constants and receptor selectivity ratios.

25 2', 6'-dimethyltyrosine (Dmt) may be substituted for tyrosine in the opioid peptide
 H-Tyr-D-Arg-Phe-Phe-NH₂. The presence of the 2', 6'-dimethyltyrosine residue in the
 peptide increases its selectivity for the μ receptor by up to two orders of magnitude. Selectivity for the μ -receptor increases when the

compound includes Dmt at the R₁ position. This substitution causes a corresponding shift in the ratio of binding inhibition constants to reflect the increased μ -receptor selectivity.

Many of the compounds listed in Table 1 show good μ -receptor binding but weak analgesic effect in the mouse writhing assay. This anomaly may be due to rapid proteolysis, rapid clearance, or both. For example, when the prototype lipophilic demorphin peptide TAPP (BCH1774) was exposed to brushborder kidney membranes, it was observed to be degraded within 15-30 minutes. Of the peptides listed in Table 1, three preferred compounds, other than TAPP itself, exhibit an increased analgesic effect *in vivo*. These three compounds are H-Tyr-D-Nva-Phe-Phe-NH₂ (BCH2462), H-Tyr-D-Ser-Phe-Phe-NH₂ (BCH2463), and H-Tyr-D-Arg-Phe-Phe-NH₂ (BCH2687). BCH2462, BCH2463, and BCH2687 have been shown to exhibit peripheral analgesia. No central analgesic effect was observed using these peptides even at doses of 100 mg/kg in the mouse hot plate test.

As shown in Table 1, the ED₅₀ value for TAPP (BCH1774) is 1.4. The corresponding values for H-Tyr-D-Nva-Phe-Phe-NH₂ (BCH2462), and H-Tyr-D-Ser-Phe-Phe-NH₂ (BCH2463), and H-Tyr-D-Arg-Phe-Phe-NH₂ (BCH2687) are 2.7, 0.5, and 0.5 respectively. The ED₅₀ values for the remaining compounds in Table 1 are higher than these figures. Although the ED₅₀ value of BCH2813 was only 0.15, it was found to act centrally at doses of about 40 mg/kg in the hot plate test.

These results indicate that the compounds BCH1774, BCH2462, and BCH2463 still undergo proteolysis but they have a longer half life and therefore are more effective as analgesic agents. In Figure 6, the duration *in vivo* of analgesic effects caused by BCH1774 (TAPP) and BCH2463 (TSPP) were compared. Using 30 mg/kg s.c. of BCH2463 and 20 mg/kg s.c. of BCH1774, Figure 3 indicates that the analgesic effect of BCH1774 lasted longer than for BCH2463, possibly indicating a slightly accelerated *in vivo* proteolysis of BCH2463 than for BCH1774.

Tables 1-5 show the effects of morphine, the μ -opioid receptor agonist, and the μ -opioid receptor antagonist, BCH2687 determined by evaluating the reaction of the mice in the hot plate test. As shown in

Figure 1A, the reaction time of the mice treated with 10 mg/kg of morphine is approximately 17 seconds. The reaction time of the mice treated with 100 mg/kg of BCH2463 (Figure 1B) is about 9 seconds compared to a control value of approximately 7 seconds. These results indicate that while morphine inhibits the nociceptive thermal stimulus, BCH2463 does not; but BCH2463 is a potent analgesic agent as is shown by the inhibition of chemically-induced writing (Figure 2). The reaction time of the mice treated with BCH2462 and with BCH2687 (Figure 1C and Figure 1D) is approximately 8 seconds.

The effects of inhibition of proteolytic metabolism of BCH2463 by the inhibitor DL-thiorphan has been studied and also the metabolic breakdown of BCH2463 mediated by brush border kidney membranes. The data obtained indicate that the kidney may be the principal site of clearance and metabolism for the compound BCH2463. From Figure 2, it appears that the endopeptidase enzyme EC24-11, which is inhibited by DL-thiorphan, is the preliminary mediator of BCH2463 proteolysis by brush border kidney extract.

Both BCH1774 (TAPP) and BCH2462 (TNPP) exhibited lethal effects upon mice when administered at 1-5 mg kg⁻¹ i.v. bolus dose of drug. In contrast, BCH2463 (TSPP) surprisingly did not exhibit any lethal effects at doses up to 20 mg/kg i.v. In addition, peptides were safe when administered subcutaneously (s.c.) at doses greater than 100 mg/kg. Thus, the structural paradigm exemplified by BCH1774 can be modified while maintaining exclusion from the central nervous system even at doses as high as 100 mg kg⁻¹ s.c. and toxicity can be minimized.

20 C. Compounds of Formula B

In a second aspect, the compounds of the present invention are defined as set forth by formula B and the associated definition of terms as set forth in the Summary section above. Compounds of the invention include but are not limited to:

Compound #1B	H-Tyr-D-Ala-Phe(pF)-Phe(pF)-NH ₂ ;
25 Compound #1C	H-Tyr-D-Ala-Phe(pF)-Phe-NH ₂ ;

In a preferred embodiment, the compounds of the invention are selected from the group consisting of:

Compound #1C	H-Tyr-D-Ala-Phe(pF)-Phe-NH ₂ ; and
Compound #2C	H-Tyr-D-Arg-Phe(pF)-Phe-NH ₂ .

In the most preferred embodiment, the compound of the invention is

5 Compound #1C H-Tyr-D-Ala-Phe(pF)-Phe-NH₂.

As with the compounds of formula (A), the amino acid derivative 2',6'-dimethyltyrosine (Dmt) may be substituted for tyrosine in the opioid peptide compounds of formula B.

D. Analgesic Activity of the Compounds of Formula B

10 PBQ (phenyl- ρ -benzoquinone) induced writhing in mice is an assessment of both central and peripheral analgesia (see Sigmund, *et al.*, *Proc. Soc. Exp. Biol. Med.* 95:729 (1957)). Central analgesia is determined by the inhibition of a hot-plate response in mice (see, Wolfe, *et al.*, *J. Pharmacol. Exp. Ther.* 80:300 (1944)). Assays measuring opioid receptor binding affinities for μ and δ receptors as well as the GPI assay were determined through experimental protocol set out in Schiller *et al.* (*Biophys. Res. Commun.* 85:1322 (1975)). The references by Sigmund, *et al.*, Wolfe, *et al.*, and Schiller, *et al.* are each hereby incorporated by reference.

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Comparison of the activities of compounds of the invention in the GPI, writhing, hot-plate, and tail flick assays demonstrated that the analgesic effects were predominantly mediated in the periphery. Peripheral analgesia was shown by a high potency in the writhing test coupled with a low potency in the hot-plate test -or the tail flick test.

E. Synthesis of Compounds of Formula A and B

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The compounds of the present invention can be produced by methods well known in the art of peptide chemistry see, *e.g.*, Principle of Peptide Synthesis, Bodansky M., Spinger-Verlag, New York, (1984); The Peptides, Analysis, Synthesis, Biology, Gross and Meienhofer eds,

Commercially available parafluoro-phenylalanine (Phe(pF)) was employed at the appropriate step of synthesis in Examples 5 and 6. 2',6'-dimethyltyrosine may also be incorporated in the synthesis and is prepared according to established chemical synthesis techniques.

5 Pharmaceutically acceptable salts of the peptides of this invention may be formed by reaction with an appropriate acid. Suitable acid addition salts may be formed by the addition of acids such as hydrochloric, hydrobromic, phosphoric, acetic, fumaric, salicylic, citric, lactic, mandelic, tartaric, oxalic, methanesulphonic, and other suitable acids known to persons skilled in the art.

10 F. Pharmaceutical Compositions

The present invention also provides for pharmaceutical compositions. Suitable compositions have a pharmaceutically effective amount of one or more peptides of the invention, or pharmaceutically acceptable salts thereof, in admixture with a pharmaceutically acceptable carrier or adjuvant. Formulations may be prepared using methods that are standard in the art (*see* 15 *e.g.*, Remington's Pharmaceutical Sciences, 16th ed., A. Oslo Editor, Easton PA (1980)). Specific dosage forms include tablets, pills, capsules, powders, aerosols, suppositories, skin patches, parenterals, and oral liquids including oil aqueous suspensions, solutions and emulsions. Sustained release dosage forms may also be used.

The peptides may be used in conjunction with any of the vehicles and excipients 20 commonly employed in pharmaceutical preparations, *e.g.*, talc, gum arabic, lactose, starch, magnesium stearate, cocoa butter, aqueous or non-aqueous solvents, oils, paraffin derivatives, glycols, etc. Coloring and flavoring agents may also be added to preparations. Solutions can be prepared using water or physiological compatible organic solvents such as ethanol, 1,2-propylene glycol, polyglycols, dimethyl sulfoxide, fatty alcohols, triglycerides, partial esters of glycerine 25 and the like. Parenteral compositions may be prepared using conventional techniques and include

G. Method of Treating of Pain

The present invention also provides for a method of treatment of pain in mammals, including humans. The method comprises administering a pharmaceutically effective amount of TAPP, a peptide of formula A, a peptide of formula B, or a pharmaceutically acceptable salt or composition thereof to a mammal in need of pain relief. The peptides can be administered to a human patient in a dosage of about 0.01 to 100mg/kg, more preferably at about 0.05 to 20 mg/kg, and most preferably at about 0.1-1 mg/kg.

Any route of administration and dosage form is compatible with the treatment method and a peptide may be administered as either the sole active agent or in combination with other drugs. Preparations may be administered epidurally, orally, perorally, internally, rectally, nasally, vaginally, lingually, sublingually, transdermally, intravenously, intraarterially, intramuscularly, intraperitoneally, intracutaneously and subcutaneously.

EXAMPLES

Example 1: Solid Phase Peptide Syntheses of Opioid Peptides of Formula A

Synthetic peptides were prepared using "RINK" resin, 4-(2', 4'-dimethoxy-phenyl-Fmoc-aminomethyl)-phenoxy resin (Novabiochem or Advanced Chemtech) and the relevant C-terminal N α -Fmoc-L-Amino acid residue of each peptide to be synthesized.

All L- and D-amino acids had their alpha group Fmoc-protected (9-fluorenylmethyloxycarbonyl) and the following side chain protection groups: t-butyl ether (tBu) for serine, threonine and tyrosine; t-butyl ester (OtBu) for aspartic acid and glutamic; t-butyloxycarbonyl (tBoc) for lysine and 2,2,5,7,8-pentamethylchroman-6-sulphonyl (pmc) for arginine and trityl (trt) for cysteine.

Dimethylformamide (Anachemia, dimethylamine-free purity) was treated with activated 4 Å molecular sieves. Piperidine (Advanced Chemtech) was used without further purification.

(Advanced Chemtech, respectively).

Solid phase peptide synthesis was carried out manually on "RINK" resin, 4-(2', 4'-dimethoxy-phenyl-Fmoc-aminomethyl)-phenoxy resin. Loading was approximately 0.6 mmol/g. Peptide condensation was carried out using the steps of: 1) Coupling: two equivalents each of Fmoc-amino acid, HOBt and DCC in DMF for 1-4 hours at room temperature; 5 2) Recoupling: one equivalent each of Fmoc-amino acid, HOBt and DCC; 3) Acetylation: 20% (v/v) (CH₃CO)₂O/DCM for 1 hour at room temperature; and 4) N- α -Fmoc deprotection: 20% (v/v) piperidine in DMF for 25 minutes.

The removal of side chain protecting groups (tBu, Boc, Trt, Pmc) and cleavage of peptide from the resin were accomplished using a TFA containing cocktail ((v/v) 55/5/40 10 TFA/anisole/DCM) for 90 minutes at room temperature under N₂. The peptide was precipitated from diethyl ether, filtered and dried. The crude peptide was purified and analyzed by HPLC on a reverse phase column with a gradient elution using 0.06% TFA/H₂O and 0.06% TFA/acetonitrile.

Example 2: Hot Plate Assay of Compounds of Formula A

15 *Measurement of Analgesic Activity*

For this test, CD #1 male mice weighing between 20 and 25g were used. The mice were weighed, marked, and divided into groups of 10.

The mice were usually treated by subcutaneous injection of the compound, the standard, or the medium in an injection volume equivalent to 0.1 ml/10g p.c. (10ml/kg). If an antagonist 20 such as Nalaxone or N-methyl-Levallorphan was used, it was administered intra-peritoneally 20 minutes before the compound (or the standard, or the medium) was administered. The injection volume was also 0.1 ml/10g p.c. The dose of the antagonist was 10 mg/kg.

The mice were individually evaluated for reaction time on the hot plate. The temperature of the hot plate (Sorel, model DS37) was set at 55°C. Mice were observed for signs of remaining on the hot plate. The reaction time was counted when the first signs appeared and was given in "seconds." Each mouse was observed for a maximum period of 30 seconds so as to prevent

damage to paw tissue. The mice may be observed at different time intervals after administration of the compound, medium or standard, *e.g.*, at 30, 60 or 120 minutes.

For each time reading, the average reaction time of the control group was multiplied by 1.5. The reaction time of each treated mouse was compared to the "control average X 1.5." If the reaction time was inferior to the "control average X 1.5," the mouse was considered to not have had an analgesic effect. If the reaction time was superior to the "control average X 1.5" then the mouse was considered to have had an analgesic effect. The number of analgesic mice in a group determined the analgesic percentage of the compound for this reading. If the analgesic percentage was lower than 30%, the compound was considered inactive.

Example 3: Writhing Assay of Compounds of Formula A

Measurement of Contortions

The test was performed on CD #1 male mice weighing between 18 and 22g. Mice were weighed, marked and then injected, by the intraperitoneal route, with 0.3ml/20g by weight of a solution of phenylquinone at 0.02%. The contortions which appeared during a 15 minute time period following the injection were counted. The phenylquinone was injected at time intervals of 5, 20 or 60 minutes after administration of the compound (or medium, or standard) by subcutaneous route. It was injected at time intervals of 60 minutes after the administration of the compound (or medium, or standard) by oral route;

The 0.02% phenylquinone solution was prepared as follows: 20mg of phenylquinone was dissolved in 5ml ethanol 90% (Sigma). The dissolved phenylquinone was slowly added to 95 ml of distilled water continuously shaken and preheated (not boiled). The phenylquinone solution was, at all times protected from light and a new solution was prepared every day for the test. Each group usually contained 10 mice. If an antagonist, such as naloxone, was used, it was administered 20 minutes before the compound (or the medium, or the standard) by the intraperitoneal route.

Table 1

BCH#	Sequence	Ki ^a [nM]	Ki ^b /Ki ^a	GPI(IC ₅₀) [nM]	ED ₅₀ (PBQ) mg/kg(20min)	Hot Plate mg/kg
1774	.r-D-Ala-Phe-Phe-NH ₂	1.53	409	3	1.4	>100
753	.r-D-Phe-Phe-Phe-NH ₂	3.63	37.7	247	>20	
754	.r-Aib-Phe-Phe-NH ₂			73	>20	
755	.r-D-Nle-Phe-Phe-NH ₂	0.968	373	15	2.5 (5 min.)	
756	.r-Pro-Phe-Phe-NH ₂	4.10	182	15	>20	
757	.r-D-Ala-Phe-2'-Nal-NH ₂	0.655	119	2	1.1 (5 min.)	
758	.r-D-Ala-2'-Nal-1'-Nal-NH ₂	5.61	102	-	>20	
1775	.r-D-Ala-D-Phe-Phe-NH ₂	26.0	82.7	925		
1776	.r-D-Ala-Phe-Phe(4-NO ₂)-NH ₂	0.509	129	8	4	
1777	.r-D-Ala-Phe(4-NO ₂)-Phe(4-NO ₂)-NH ₂	0.826	570	6	>20	
1778	.r-D-Ala-Phe-Phe(4-N ₃)-NH ₂	1.49	107	50		
1779	.r-D-Ala-Phe(4-NO ₂)-Phe-NH ₂	56.8	24.3	77		
1780	.r-D-Ala-Phe-Tic-NH ₂	12.7	279	-		
1781	.r-D-Ala-Phe-Phe(NMe)-NH ₂	22.6	215	241		
1782	.r-D-Ala-Phe-1'-Nal-NH ₂	0.981	174	2	>20	

BCIH#	Sequence	Ki ^u [nM]	Ki ^b /Ki ^u	GPI(IC ₅₀) [nM]	ED ₅₀ (PBQ) mg/kg(20min)	Hot Plate mg/kg
1783	L-D-Ala-1'-Nal-1'-NH ₂	2.88	410	-	>20	
1784	L-D-Ala-Trp-Phe-NH ₂	3.57	238	20	>20	
1785	L-D-Ala-Phe-Trp-NH ₂	2.21	214	16	>20	
1786	L-D-Ala-Trp-Trp-NH ₂	0.833	783		10	
1787	L-D-Ala-Phe-Phe-NH ₂				10	
2202	L-Tyr-D-Ala-Phe-Phe-NH ₂				>10	
2208	L-D-Nle-Phe-Trp-NH ₂				>3	
2211	L-D-Nle-Phe-2'-Nal-NH ₂				>10	
2212	L-D-Nle-Trp-Phe-NH ₂				>10	
2213	L-D-Ala-Trp-2'-Nal-NH ₂				>5	
2214	L-D-Nle-Trp-2'-Nal-NH ₂				15	
2217	L-D-Nle-Trp-Trp-NH ₂				>5	
2462	L-D-Nva-Phe-Phe-NH ₂				2.7	>100
2463	L-D-Ser-Phe-Phe-NH ₂	2.2		13	0.5	>100
2464	L-D-Val-Phe-Phe-NH ₂				>10	
2465	L-D-Leu-Phe-Phe-NH ₂				>10	

BCH#	Sequence	Ki ^u [nM]	Ki ^b /Ki ^u	GPI(IC ₅₀) [nM]	ED ₅₀ (PBQ) mg/kg(20min)	Hot Plate mg/kg
2473	r-D-Ile-Phe-Phe-NH ₂				>10	
2577	r-D-Abu-Phe-Phe-NH ₂				>10	
2578	r-Chl-Phe-Phe-NH ₂				>10	
2579	r-Cle-Phe-Phe-NH ₂				>10	
2687	r-D-Arg-Phe-Phe-NH ₂	0.88	2480	8.71	0.5	>100
2690	r-D-Cys-Phe-Phe-NH ₂				6.2	>100
2811	r-D-Thr-Phe-Phe-NH ₂				12	
2813	nt-D-Ser-Phe-Phe-NH ₂				0.15	-40
3237	nt-D-Ala-Phe-Phe-NH ₂	0.16	26.4	0.34		
3238	nt-D-Ala-Phe-Phe-NH ₂	71.8	3.1			
3240	r-D-Ala-Phe-Cys(Bzl)NH ₂	5.3	57.3	9.16		
3241	r-D-Arg-Phe-Cys(Bzl)NH ₂	6.95	46.8	33.05		

Example 4: Preparation of H-Tyr-D-Ala-Phe(pF)-Phe-NH

The synthetic peptide was prepared using Knorr resin. The amino acids used had their alpha amino group Fmoc-protected and Tyrosine side chain tBu protected. Dimethylformamide used in the coupling step was free of dimethylamine. DNT used for the washing steps and TFA were Biograde purity. For the purification step USP purified H₂O and acetonitrile of HPLC grade were used. All remaining solvents were of ACS purity and used as such without any purification. Solid phase synthesis was carried out manually on the resin having a load of 0.84 mM/g. Peptide condensation was carried out using 1.5 to 2 equivalents each of Fmoc-amino acid, HOBT and BOP in DMF for 3-24 hours at room temperature. The alpha amino Fmoc deprotection steps were performed using 20 % (v/v) Piperidine in DMF for 25 minutes. The peptide cleavage and side chain deprotection were accomplished by treatment with TFA/CH₂Cl₂/anisole. The peptide resin was treated with TFA for two periods of 90 minutes at room temperature under nitrogen atmosphere. After CH₂Cl₂ washing and evaporation the residue was treated with ethyl ether, the precipitate filtered and dried under vacuum.

The crude peptide obtained was purified by HPLC on a C₁₈ 10μ-15μ 300A reverse phase column, with a gradient elution of 0.06 % TFA/H₂O to 0.06% TFA/Acetonitrile. Monitoring was performed at 220 nm. Pure fractions were pooled and lyophilized. The purified material was exchanged into its hydrochloride salt form to give the pure title compound.

In a like manner the following peptides were also synthesized:

- 1A H-Tyr-D-Ala-Phe-Phe-NH₂
- 1B H-Tyr-D-Ala-Phe(pF)-Phe(pF)-NH₂

Example 5: Preparation of H-Tyr-D-Arg-Phe(pF)-Phe-NH₂

The synthetic peptide was prepared using Knorr resin. The amino acids used had their alpha amino group Fmoc-protected. Side chains were protected using Pmc for D-Arginine, and tBu for Tyrosine. Dimethylformamide used in the coupling step was free of dimethylamine. DMF and acetonitrile of HPLC grade were used. All remaining solvents were of ACS purity and used as such without any further purification.

Solid phase synthesis was carried out manually on the resin with a load of 0.84 mM/g. Peptide condensation was carried out using 2 equivalents each of Fmoc-amino acid, HOBT and BOP in DMF for 2-5 hours at room temperature. The alpha amino Fmoc deprotection steps were performed using 20% (v/v) piperidine in DMF for 25 minutes. Peptide cleavage and side chain deprotection were accomplished by TFA/CH₂Cl₂/anisole treatment. The peptide resin was treated with TFA for two periods of 90 minutes at room temperature under nitrogen atmosphere. After CH₂Cl₂ washing and evaporation the residue was treated with ethyl ether. The precipitate was then filtered and dried under vacuum.

The crude peptide obtained was purified by HPLC on a C₁₈ 10μ-15μ 300A reverse phase column, with gradient elution using from 0.06% TFA/H₂O to 0.06% TFA/acetonitrile. Monitoring was performed at 220 nm. Pure fractions were pooled and lyophilized.

In a like manner the following peptide compounds were synthesized:



15 **Example 6: Radioligand Binding Assay of Compounds of Formula B**

Membrane Preparation

Male Sprague-Dawley rats weighing between 350-450g were sacrificed by inhalation of CO₂. The rats were decapitated and the brains (minus the cerebellum) were removed, placed in ice-cold saline solution, and then homogenized in ice-cold 50 mM Tris buffer pH 7.4 (10ml/brain). Membranes were centrifuged at 14000 rpm for 30 min. at 4°C. The pellets were re-suspended in approximately 6ml/brain of ice-cold Tris buffer 50mM pH 7.4 and stored at -78°C until ready for use. Protein quantification of the brain homogenate was conducted according to protein assay kit purchased (Bio-Rad).

Radioligand Inhibition

Receptor membranes and membranes containing certain related cell components were incubated for 1 hr at 22°C. Nonspecific binding was determined using a 500 fold excess of

unlabeled ligand in the presence of tracer and membranes. Free ligand was separated from bound by filtration through Whatman GF/B paper (presoaked in polyethylenimine 1% aqueous solution) and rinsing with ice-cold 50mM Tris pH 7.4 using a Brandel cell harvester. The filters were dried and radioactivity was counted in a 24 well microplate in the presence of 500 μ l scintillant per well. Radioactivity was measured using a Wallace 1450 Microbeta counter. K_i 's for the various compounds were determined from the IC_{50} according to the Cheng and Prusoff equation. Results of the binding assay are summarized in Table 2.

The activity of the peptide compounds on μ receptors was determined using the Guinea Pig Ileum (GPI) assay (longitudinal muscle preparation) according to the procedures described in Schiller *et al.*, *Biophys. Res. Commun.* 85:1322 (1975). Activity results are summarized in Table 2.

Example 7: Hot Plate Assay of Compounds of Formula B

For hot plate assays, CD #1 male mice weighing between 20 and 25g were used. The mice were weighed, marked, and divided into groups of 10. Mice were treated by subcutaneous injection of the compound (or the standard or the medium) in an injection volume equivalent to 0.1ml/10g (10ml/kg). The remaining conditions of the experiment and calculations were as described in Example 2. Results are shown in Figures 4 to 6.

Example 8: Writhing Assay of Compounds of Formula B

Writhing assays were performed on CD#1 male mice weighing between 12 and 22g. Assays were carried out as described in Example 3 and results are summarized below in Table 2. It can be seen that the peptides in which either one or both of R_3 and R_4 are Phe(pF) exhibit greater selectivity for the μ opioid receptor compared to the corresponding compound without Phe(pF). In addition, these peptides exhibit a greater transduction of the receptor as determined in the GPI assay and greater peripheral analgesic activity as determined in the writhing assay.

Table 2

Peptide	Binding Assay			Writhing	CPI
	K ₁ μ (nM)	K ₁ δ (nM)	K ₁ δ K ₁ μ	ED ₅₀ (mg/kg)	IC ₅₀ (nM)
1A	1.53	625.8	409	1.4	3
1B	0.2	199.6	998	0.2	0.12
1C	0.36	201.2	559	0.5	
2A	0.68	1652.6	2430	0.5	6.7
2B	0.22	>1000		0.3	
2C	0.57	952.5	1671	0.3	1.52

Example 9: 58°C Hot Plate Assay of Compounds of Formula B

10 For this assay, NMR1 male mice weighing between 20 and 25g were used. The mice were weighed, marked, and divided into groups of 6. They were then treated by subcutaneous injection of the compound (or the standard or the medium) in an injection volume equivalent to 0.1 ml 10g p.c. (10ml/kg).

15 The mice were individually evaluated for reaction time on the hot plate. The temperature of the hot plate (IITC, Inc; Model 35-0) was set at 58°C. The mouse was observed for signs of discomfort such as licking or shaking of the paws, attempting to escape (jumping off the plate) or trembling. The reaction time was counted when one of these signs appeared and was noted in "seconds". Each mouse was observed for a maximum period of 20 seconds so as to prevent damage to the paw tissue.

20 The compound was considered analgesic if the reaction time was significantly different ($p < 0.05$; two way ANOVA, sigma slot) from the control group. Results are shown in Figure 7.

25 weighed, marked, and divided into group of 6. The mice were treated by subcutaneous injection of

the compounds (or the standard medium) in an injection volume equivalent to 0.1 ml/10g p.c (10 ml/kg).

Mice were individually evaluated for reaction time in the tail flick test. The latency to the flick of the tail was measured when a rheostat-controlled light beam was directed at the tip of the tail (HTC Inc. Model 33). Each mouse was observed for a maximum period of 10 seconds so as to prevent damage to the tissue. Results are shown in Figure 8.